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# Inducer line generated double haploid seeds for combined waxy and opaque 2 grain quality in subtropical maize (*Zea mays.* L.)

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**Abstract** The implementation of modern inducer lines in maize breeding can substantially decrease the time required to create elite inbred lines. In industrialized countries, this method has already largely replaced conventional backcross methods. However, the application of in vivo gynogenesis for inducing doubled haploids is still limited to European and US maize germplasms and has still to be adapted for exotic plant material. The reliability of three modern European inducer lines from the University of Hohenheim (Germany) was investigated for the production of haploid progenies from subtropical specialty maize. Three Chinese hybrids heterozygous for waxy maize and opaque 2 were used as maternal donor material, as maize double recessive for waxy and opaque 2 may improve the nutrition of ethnic minorities in Southeast Asia. However, many false positives were detected by flow cytometry among putative haploid seeds based on anthocyanin pigmentation because the color expression was inhibited in almost 50% of the induced seeds from this maternal plant material. Based on flow cytometry, the haploid induction rates were high with 10.2–12.3%, and the chromosome doubling rates were around 50%; therefore the principal potential of producing DH was confirmed for subtropical maize. However inducer lines for the precise and fast recognition of truly induced haploid seeds still need to be developed.

**Keywords** Doubled haploid · Inducer lines · Quality protein maize · Waxy maize

**Abbreviations**

| | |
|---|---|
| *R1-nj* | R-navajo |
| *C1* | Colored |
| *c1* | Uncolored |
| $C1^I$ | Colored inhibitor |
| FCM | Flow cytometry |
| ASI | Anthesis silking interval |

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## Introduction

Haploid maize plants can be produced in vitro or in vivo. In in vivo, maize haploids are produced mostly by crossing the genotypes of interest with specific inducer genotypes, the so-called inducer lines. Compared to in vitro techniques, in vivo haploid induction

procedures appear to be easier and more reliable, which does not mean that they are not still improvable. In vivo haploid induction has been improved drastically in the last 30 years by more efficient inducer lines with increasing induction rates from around 2.3% in the early 60's (Coe 1959) to 3–5% (Lashermes and Berkert 1988) and 6% (Sarkar et al. 1994) in the 90's to up to more than 8% with the modern European line RWS (Röber et al. 2005). The key issue in applying in vivo haploid induction is the selection of haploid seeds among the progeny. To facilitate the selection of haploid seeds, modern inducer lines carry the *R1-nj* gene, which causes a "red crown" endosperm as well as a red (or purple) embryo. After pollination by inducer lines, haploid seeds combine a haploid maternal embryo (colorless) and a triploid endosperm (red crown aleurone) derived from both, the maternal donor and the inducer line (Geiger 2009). However, the anthocyanin pigmentation is influenced by the environment and the genetic background of the donor plant, which renders it a very complex and sometimes unreliable system (Cone 2007). The *C1* gene plays a regulatory role in the production of anthocyanin pigments. As well as *C1*, a couple of other genes such as *R1*, and *Bz1,* were reported to be interacting with *C1* to form anthocyanin pigments (Cone 2007). The complex regulation of the anthocyanin production system seems dependent on two other genes/alleles, the $C1^I$ gene, which acts as an inhibitor of *C1*, and the *c1* recessive gene, which does not induce pigmentation of the maize aleurone.

Since haploid plants are sterile, doubling the chromosome set becomes the most important step after having selected haploid seedlings. The frequency of spontaneous fertility of the female inflorescence in haploid maize is quite high, ranging from 25.0 to 96.0%, while spontaneous male fertility occurs much less frequently (Chang and Coe 2009; Geiger 2009). In maize, artificial chromosome doubling by treatment with colchicine has enabled the successful self-pollination of plants, which were originally haploids (Chase 1952). The doubling rate of tassels of haploid plants treated with a solution of 0.06 to 0.50% colchicine reached up to 50%. The lowest concentration was best suited as higher colchicine concentrations were rather deleterious for seedlings causing severe injury and very low recovery rates (Han et al. 2006). Kato (2002) used nitrous oxide gas ($N_2O$) on maize at the 6th leaf stage, increasing the doubling rate to up to 44%, but this method is very expensive and requires special facilities. Therefore colchicine is still the most efficient chemical to double the chromosome set in maize but its efficiency and ideal concentration are genotype-dependent; no common method could achieve the best result for all genotypes (Castillo et al. 2009).

In Southeast Asia, the specialty waxy maize is consumed as the staple food for many minority ethnic groups in the uplands; in lowland areas it is often cultivated as an additional vegetable. Waxy maize has almost pure amylopectin, controlled by a single recessive *wx* allele of the *waxy* gene, located on the short arm of chromosome 9 (Coe et al. 1988). The protein in waxy maize, like that of regular maize, is low in quantity and poor in quality; it is particularly deficient in lysine and tryptophan, which are the most important amino acids, especially for infants (WHO 1985). Therefore the consumption of waxy maize as a staple food in Southeast Asia often leads to malnutrition. The protein of other specialty maize with the mutation genes *opaque2* (*o2*) and/or *floury2* (*fl2*), is of much better quality (Mertz et al. 1964). Negative side impacts of such genes on the physical properties of the grain were overcome by specific modifier gene(s) for *o2*. This has led to the production of *o2* kernels, that are particularly hard, and named high quality protein maize (QPM) (Prasana et al. 2001). In the last few decades, QPM has become an important part of maize breeding programs in many developing countries.

Doubled haploid production is a promising approach to obtain rapidly homozygous plants for maize breeding programs, but its application to tropical and subtropical waxy maize is still very limited and little in use. The efficiency of the haploid induction seems to be very variable, depending on the inducer line but also on the maternal plant material, which plays a determining role in the success of the haploid induction.

The objectives here were, first, to find the best method for identifying haploid seeds derived from tropical and subtropical waxy × QPM hybrids after haploid induction with different modern European inducer lines and second, to determine the doubling and fertility of these haploid plants.

## Materials and methods

### Plant material

Three inducer lines, RWS, RWK76, and RWK76 RWS, kindly provided by Prof. H.H. Geiger, University of Hohenheim, Germany, were used to generate haploid plant material. RWS and RWK76 were derived from the cross between the Russian inducer line KEM and the French WS14 (Geiger and Gordillo 2009; Röber et al. 2005). RWK76RWS was the hybrid between these two inducer lines RWS and RWK76. The RWS inducer line was smaller, had a smaller tassel and produced less pollen than RWK76 and the hybrid RWK76RWS. In addition to the *R1-nj* marker gene, all carried a sun-independent gene (*Pl1*), the expression of which leads to a red colored stem at the seedling stage. This should allow breeders to select easily the haploid plants out of false positive cases. Three waxy × QPM hybrids derived from crosses between waxy and QPM lines (i.e. heterozygous for both genes), which were used as donor parents: CN3, CN35, CN37 from the Guangxi Maize Research Institute, China. These hybrids were hand-pollinated with pollen of the inducer lines to generate haploid seeds in the greenhouse. The resulting seeds were used to evaluate and optimize the selection method for haploid seeds of this subtropical germplasm. The selected haploid populations were referred to ETH3, ETH35, and ETH37.

### Selection of haploid seeds

#### *Visual selection according to anthocyanin pigmentation*

The seeds obtained after induction were divided into three bulks: (1) unpigmented seeds (unpigmented endosperm and unpigmented embryo), (2) *putative* haploid seeds (red endosperm, unpigmented embryo) and (3) diploid seeds (red endosperm and red embryo). All seeds with pigmented embryos were considered as diploid seeds ($F_1$ hybrid between the maternal hybrid and the inducer line). Only seeds with unpigmented embryos, bulk 1 and bulk 2, were considered for further testing.

The three modern inducer lines carry the sun-independent (*Pl1*) gene, resulting theoretically in an exclusive diploid—induced progeny with a red stem whereas the stem of haploid seedlings remains green. This should help to identify haploid plants, where the classical anthocyanin selection is unreliable.

#### *Flow cytometry*

Flow cytometry (FCM) allows one to identify effectively haploid seedlings among the induced progeny. The selection of haploid seedlings is only the first step in the process of developing DH, and thus, it must be non-destructive. The ploidy of *putative* haploid seeds (bulk 2) as well as of unpigmented seeds (bulk 1) was evaluated after germination (3–4 days incubated at 28°C). The small tip of the first leaf (right after emergence from the coleoptile) was cut off, chopped into small pieces with a razor blade and stained with 2 ml DAPI (4′,6-diamidino-2-phenylindone) solution (5 µg/ml, Partec GmbH, Germany). After filtering through a nylon membrane (50 µm mesh size), the filtrate was analyzed by FCM, at a par gain FL1 (fluorescence) of 420–430 nm (relative fluorescence—RF). By a peak set at 60 and 130 FL (corresponding to nuclei in growth phase1 (G1) and growth phase2 (G2), respectively) diploid material was identified, while a peak set at 30 and 70 FL, respectively corresponded to haploid plant material.

### Chromosome doubling

Chromosome doubling of the selected haploid seedlings was done according to Deimling et al. (1997). The entire coleoptiles of seedlings (4–5 days after germination) were immersed in a solution of 0.06% colchicine and 0.05% DMSO (dimethyl sulfoxide) for 12 h. Thereafter, the coleoptiles were washed under running tap water for 20 min and the seedlings were grown in pots under greenhouse conditions. Two to three weeks after the treatment, leaf samples from the youngest leaf were collected to control the ploidy level of the treated plants by flow cytometry and thus, to assess the success of chromosome doubling.

### Statistics

Analyses of variance (ANOVA) of the induction and doubling rates were performed using the MIXED procedure of the SAS® 9.1.3 software (SAS Institute Inc. 2004). The replicates were treated as random factor and waxy × QPM donor hybrids, inducer lines

and their interaction as the fixed effects. Sources of variation and appropriate F ratios (Type III) were applied according to McIntosh (1983). For the comparison of means estimates a Student Newman–Keuls test (*t* test) was chosen as it has less risk of type 1 errors than other tests like Duncan's multiple range, Tukey-compromise, Scheffe's S, Games/Howell. Therefore, the latter tests may cause some loss of robustness. Standard deviation and confidence interval were performed using the MEANS SAS® 9.1.3 software. The MEANS procedures here were used to compute simple statistics. Besides, the other procedures in SAS for analysis, such as ANOVA, MIXED and GLM procedures were also used and avoid loss of information provided by the analyses of variance.

## Results

### Selection of haploid seeds after induction

After haploid induction, 1842 induced seeds were harvested and separated into three bulks according to their aleurone and the color of the embryo. The expression of anthocyanin (red crown) was inhibited or over-expressed depending on the maternal genotype. Seeds with pigmented embryos (792 seeds, 43% of the induced seeds) were identified as diploid $F_1$ hybrids between the donor hybrid and the inducer line and no longer taken into consideration. The main issue was to eliminate false positives (unpigmented embryo but nevertheless diploid) present in the two bulks: bulk 1 seeds (712 seeds, 38.7% of the induced seeds) where both the embryo and the endosperm were unpigmented (unpigmented seeds) and bulk 2 seeds (338 seeds, 18.3% of the induced seeds) of the unpigmented embryo but with a pigmented endosperm (*putative* haploid seeds). The hybrid CN35 produced almost no bulk 1 seeds (unpigmented) but it carried from 12.9 to 20.2% *putative* haploids with the typical "red-crown" aleurone after induction by the three inducer lines, indicating the successful induction. Meanwhile, the other induced hybrids produced on average progenies with about 50% unpigmented endosperm and embryos (bulk 1) (Table 1). However, the general proportion of *putative* haploids in the induced progeny (pigmented endosperm and unpigmented embryo) varied significantly from 15.0 to 23.8% with regard to the inducer line used as well as to the donor genotype. The combination inducer line and donor hybrid significantly impacted the proportion of *putative* haploid seeds among the progeny (data not shown). Moreover, the maternal donors differed significantly with regard to the rate of unpigmented seeds (bulk 1) and may play an important role in the expression of anthocyanin pigmentation. The rates of *putative* haploids among the progenies induced with the inducer lines RWK76 and RWK76RWS (15.7 and 15.0% on average, respectively) were significantly lower ($P < 0.05$) than those observed with the inducer line RWS (23.8%) for the same maternal plant material. Among the three hybrids induced with RWS, CN37 had the highest proportion of *putative* haploid seeds in the progeny (42.4%).

The ploidy level of the seeds of the two bulks with uncolored embryos, unpigmented seeds (bulk 1), and *putative* haploid seeds (bulk 2) was assessed by flow cytometry (FCM) in order to detect false positives. Quite a high number of real haploid seeds (between 2.8 and 5.5% of the large total number of induced seeds) was surprisingly found among the unpigmented seeds (bulk 1) across the three inducer lines, whereas just about half of the much smaller number of *putative* haploid seeds (bulk 2) were indeed haploids (Table 2). The proportion of real haploid seeds in the two bulks varied strongly depending on the waxy × QPM donor hybrid, but the total real haploid rates were similar for the three inducer lines.

### Chromosome doubling

Haploid seedlings selected by FCM were used for chromosome doubling. Although survival rates were high for seedlings derived from all hybrids, ranging from 47.2 to 80.0% (Table 3), the seedlings recovered slowly and exhibited unusual phenotypes (twisted leaves, dwarf plants, slower growth) at the first development stages. Seedlings of ETH35 were more robust than those from the two others maternal donors. Chromosome doubling rate was, however, just around 30% on average.

Around 50% of the plants that survived eventually had a diploid chromosome set. Again, seeds derived from CN35 exhibited the best response to chromosome doubling after the colchicine treatment with a

**Table 1** Rate (%) of unpigmented seeds (bulk 1) and of putative maize haploids seeds (bulk 2) among the induced seed progeny of three waxy*QPM hybrids, CN3, CN35, CN37, after pollination by three different inducer lines, RWS, RWK76 and RWK76RWS

| | Bulk 1 (unpigmented seeds) | | | Bulk 2 (*putative* haploid seeds) | | |
|---|---|---|---|---|---|---|
| | *RWS* | *RWK76* | RWK76RWS | RWS | RWK76 | RWK76RWS |
| CN3 | 53.5 | 48.7 | 53.3 | 10.5 | 8.6 | 6.6 |
| CN35 | 1.9 | 0.0 | 0.0 | 18.5 | 12.9 | 20.2 |
| CN37 | 51.7 | 53.4 | 45.0 | 42.4 | 24.0 | 19.5 |
| Mean | 35.8 ± *26.4*[§] | 34.9 ± *24.8* | 32.8 ± *25.4* | 23.8 ± *7.4* | 15.7 ± *7.3* | 15.0 ± *14.9* |

Rates (%) are relative to the total number of induced seeds of a specific combination of donor hybrid and inducer line

[§] Confidence interval of the average values at the 0.05 probability level are given in italic

**Table 2** Proportion of real maize haploid seeds (%) in the bulk 1 (unpigmented seeds) and in the bulk 2 (putative haploid seeds) as indicated by flow cytometry

| | Bulk 1 (non-pigmented seeds) | | | Bulk 2 (putative haploid seeds) | | | Real haploid induction % |
|---|---|---|---|---|---|---|---|
| | RWS | RWS | RWK76 | RWS | RWK76 | RWK76-RWS | |
| CN3 | 7.5[b§] | 5.2 | 6.2 | 6.0[c] | 3.0 | 5.1 | 11.0 |
| CN35 | 0.0[a] | 0.0 | 0.0 | 12.7[d] | 8.6 | 15.7 | 12.3 |
| CN37 | 10.2[b] | 4.9 | 3.8 | 5.5[c] | 3.9 | 3.4 | 10.2 |
| Mean | 5.5 ± *3.5*[#] | 2.8 ± *2.3* | 3.4 ± *2.02* | 7.4 ± *2.6* | 8.2 ± *5.4* | 6.7 ± *4.5* | |

Rates (%) are relative to the total number of induced seeds of a specific combination of donor hybrid and inducer *line*

[§] Value (in column) followed by the same letter are not different at the 0.05 probability level

[#] Confidence interval of the average values at the 0.05 probability level are given in italic

**Table 3** Survival and doubling rate (%) (relative to the total number of haploid seeds treated) after colchicine treatment of maize haploid seeds derived from three Chinese waxy*QPM hybrids

| Haploid population | Survival rate (%) | | | Doubling rate (%) | | |
|---|---|---|---|---|---|---|
| | Mean | Std Dev | CI | Mean | Std Dev | CI |
| ETH3 | 47.2[a§] | 42.7 | [2.4–92.0] | 27.8[d§] | 38.9 | [−13.1–68.6] |
| ETH35 | 80.0[c] | 23.9 | [50.3–109.7] | 54.1[f] | 24.5 | [23.6–84.5] |
| ETH37 | 73.6[b] | 41.9 | [21.6–125.7] | 35.0[e] | 25.3 | [3.6–66.4] |
| Mean | 65.7 | 38.1 | [45.4–86.1] | 38.3 | 31.1 | [21.7–54.8] |

*Std Dev* standard deviation, *CI* confidence interval at the 0.05 probability level

[§] Means (in column) followed by the same letter column are not significantly different at the 0.05 probability level

doubling rate of almost 55.0%. The main purpose of chromosome doubling is to produce homozygous diploid plants that can be multiplied by self-pollination. Therefore the number of seeds produced by these DH plants is crucial for the evaluation of the success of the whole process. In general, the fertility rate of DH plants was low; depending on the waxy × QPM hybrid of origin, it ranged from 12.5% for DH of the haploid population ETH3 to 26.5% for those of ETH37 (Fig. 1).

## Discussion

### Selection of haploid seeds after induction

In vivo gynogenesis to produce DH mostly relies on the visual marker *R1-nj* for anthocyanin pigmentation. The appearance of unpigmented induced seeds in this study illustrated that the expression of anthocyanin pigmentation may result from a genetic interaction between the inducer line (responsible for pigmentation) and the

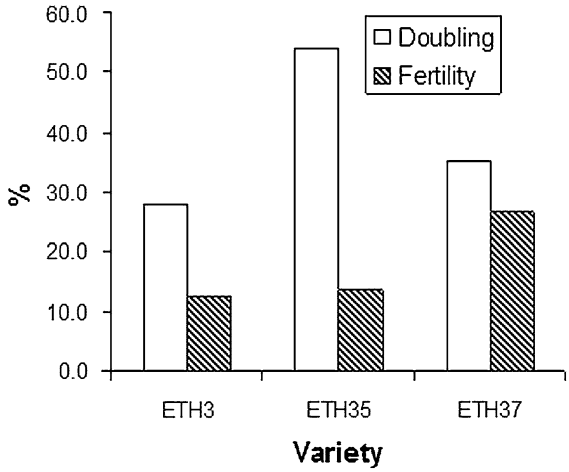


**Fig. 1** Chromosome doubling and fertility rate of haploid, respectively DH, maize plants (relative to the total number of haploid seeds treated) of five haploid populations derived from Chinese waxy × QPM hybrids

related genes of the maternal donor hybrid. This interaction was probably responsible as well for the presence of many "false positive" seeds in the bulk 2 of *putative* haploids in this subtropical waxy maize and QPM maternal germplasm. The number of real haploids eventually confirmed by FCM attested the necessity of combining two selection methods to achieve an effective selection of haploid seeds as a first step toward DH production with this plant material, a bottleneck for acceptance by breeders and necessitating to improve the system.

A relatively high rate of false positives was reported in European flint plant material as well (Röber et al. 2005) e.g. among *putative* haploid seeds after induction with RWS. Despite the failure in anthocyanin pigmentation of the aleurone of the induced seeds, haploids could still distinguished from diploid seedlings later by the red coloration of the stems as the result of the sun-independent gene (*Pl1*) carried by the inducer line. Belicuas et al. (2007) achieved up to 51% of *putative* haploid seeds by paternal haploid induction with the inducer line W23 with tropical donor material, but only about 0.9% were confirmed as real haploids. Among these subtropical waxy × QPM maternal donors, only hybrid CN35 behaved in accordance with the standard protocol of selection, i.e. in the manner of anthocyanin pigmentation. All the seeds derived from this hybrid showed a pigmented endosperm after haploid induction with all inducer lines. Although 30% of the *putative* haploid seeds were diploid and, thus, false positives, this rate remains acceptable.

The high proportion of unpigmented seeds as well as the detection by FCM of haploids among these seeds (bulk 1) illustrated that the anthocyanin pigmentation was not a reliable method for detecting haploid seeds derived from our subtropical waxy × QPM hybrid plant material, although the total proportion of real haploids was higher than the average of 8%, which is usual for European germplasm (Röber et al. 2005). Even though the extra-effort for selecting haploid seeds among the induced progeny to some extent disqualifies the technique for a fast routine application, the relatively high proportion of real haploid seeds is promising and encouraging for the implementation of inducer lines with subtropical and probably also with tropical plant material in order to generate DH for breeding.

The three inducer lines used in our study carry the *R1-nj* gene for the red crown endosperm and the red embryo. Thus, in an ideal case, a red endosperm is expected for the whole progeny as a result of the cross between the maternal donor hybrid and the inducer line. A frequency of almost 50% of unpigmented seeds among the induced seeds might be due to a heterozygous gene ($C1^{I}c1$) carried by the maternal donors, thus, inhibiting the expression of the

**Table 4** Possible combinations of the three maize alleles *C1*, $C1^{I}$ and *c1* in the parental lines and eventually in the endosperm and embryo of induced seeds with the related effect on the pigmentation (P, pigmented or N–P, unpigmented) of the triploid endosperm and the embryo (haploid or diploid)

| Inducer lines | | Maternal donors | | Endosperm | | Embryo | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Haploid | | Diploid $F_1$ | |
| *C1C1* | P | $C1^{I}$ *c1* | N–P | $C1^{I}C1c1$ | N–P | $C1^{I}$ | N–P | $C1^{I}C1$ | N–P |
| | | | | | P | *c1* | N–P | *C1c1* | P |
| | | *c1c1*[§] | N–P | *C1c1c1* | P | *c1* | N–P | *C1c1* | P |

[§] Possible situation of CN35

paternal allele *R1-nj*. After pollination by the inducer lines (*C1C1*), the progeny would then segregate in anthocyanin expression (Table 4). The exception of CN35 could then be explained by its homozygous form (*c1c1)* of this gene, resulting in a pigmented endosperm (*C1c1c1*). However, modifier genes for *o2* present in QPM may alter the "red crown" coloration of the aleurone (Becraft and Asuncion-Crabb 2000; Lopes and Larkins 1991) and result in a crown with a single red spot on the aleurone (data not shown).

### Chromosome doubling

Chromosome doubling by colchicine treatment is the most difficult step towards DH because of the high mortality and low rates of doubling. Although colchicine is a toxic chemical for living organisms, its application in DH production keeps growing as no other chemical can compete with it in terms of chromosome doubling efficiency. Doubling rates here of 28% to 54% were higher than those of Gayen et al. (1994). Our treated plants were usually weak and had a long anthesis silking interval (ASI), as already observed for their waxy × QPM donor hybrids, a main bottleneck to arrive at the selfing step successfully. Since tropical maize is very sensitive to the photoperiod and to temperature in the early vegetative stages, changing the early growing conditions to a short day with higher temperature may permit to synchronize the male and female flowerings of the DH (Gouesnard et al. 2002). The technique of chromosome doubling remains the main obstacle to the use of in vivo haploid induction in exotic waxy and QPM varieties. In order to increase the interest in the DH technique for breeding subtropical maize the implementation of alternative chemicals is highly desirable. Ongoing research seems to be promising in the last years (Geiger, personal communication 2010).

## Conclusions

Doubled haploids in general and the technique of in vivo haploid induction in particular, is a method of high potential in modern maize breeding. It has become very important in maize breeding outside of Europe and USA as well. This study was so far the first application of in vivo haploid induction with subtropical and tropical waxy and quality protein maize. Although the use of anthocyanin pigmentation for selecting the haploids after gynogenesis was unreliable because waxy × QPM plant material seemed to carry inhibitor genes, eventually high haploid induction rates achieved in this project may encourage breeders to consider the DH technique for this type of plant material. In this study, doubled haploid lines derived from different waxy × QPM donor hybrids were achieved. The next steps will be to select the double recessive *wx-o2* genotypes among the DH and to investigate their quality.

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